

# Decline of a giant salamander assessed with historical records, environmental DNA and multi-scale habitat data

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## Funding information

Pennsylvania Department of Conservation and Natural Resources Wild Resource Conservation Program. Grant Number: WRCP-014516; Bloomsburg University Research and Scholarship Grant

## Abstract

1. Freshwater species are declining rapidly but more complete data are needed for determining the extent and cause(s) of population declines and extirpations. Integrating newer survey techniques, freely available data, and traditional field work may allow for more effective assessment of population decline.
2. We used detailed historical species records and environmental DNA (eDNA) survey methods to identify changes in population distribution of a long-lived, imperiled stream salamander, the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*: Cryptobranchidae). We used logistic regression with Bayesian inference to test whether selected environmental variables may be good predictors of hellbender population persistence and extirpation.
3. Hellbenders persisted in only 42% of the 24 historical record sites. The best fit model indicated electrical conductivity (EC) was the strongest predictor of hellbender population persistence ( $EC < 278 \mu\text{S}/\text{cm}$ ) and extirpation. Conductivity was strongly negatively correlated with canopy cover within the total watershed ( $r = -0.83$ ,  $n = 21$ ,  $p < 0.001$ ) and riparian buffer of the watershed ( $r = -0.77$ ,  $n = 21$ ,  $p < 0.001$ ).
4. Electrical conductivity tends to increase following deforestation, and may inhibit sperm motility and thus limit recruitment of hellbenders and other aquatic vertebrate species with external fertilisation.
5. By integrating historical data, eDNA, field data, and freely available high resolution remote sensing data, our study design allowed for rapid assessment of predictors of and changes in hellbender distribution over a relatively broad geographic area. This cost- and time-effective approach may be used for evaluating other rare aquatic species.

## KEYWORDS

conductivity, environmental DNA (eDNA), hellbender salamander, population decline, watershed deforestation

## 1 | INTRODUCTION

Biodiversity loss in conjunction with the effects of human population growth and resource consumption has resulted in an estimated 58%

decline in global vertebrate population abundance between 1970 and 2012 (WWF, 2016). The rapid decline and loss of wildlife populations necessitate rapid population status assessment, elucidation of the often complex and interacting causal factors of decline, and

efficient and effective conservation measures (Bland et al., 2016; WWF, 2016). Many species, especially those that are cryptic, not easily accessible or surveyed, or inhabit otherwise understudied ecosystems, have been poorly studied and more complete data are needed for determining the extent and cause(s) of population declines and extirpations (Abell, 2002; Bland et al., 2016; McKinney, 1999). Freshwater species and ecosystems are particularly poorly studied (Abell, 2002). Data deficiencies exist for many freshwater species, especially those in lotic ecosystems (Collen et al., 2014; Vörösmarty et al., 2010). Available data suggest population declines and extinction rates of freshwater species dramatically surpass those of terrestrial species (Abell, Lehner, Thieme, & Linke, 2016; Abell et al., 2000; Collen et al., 2014; Dudgeon et al., 2006; Ricciardi & Rasmussen, 1999; Vörösmarty et al., 2010; WWF, 2016).

Freshwater species are imperiled by a variety of interacting factors, many of which are related to the pervasive degradation, modification, and destruction of the ecosystems in which they are found (Collen et al., 2014; Dudgeon et al., 2006; WWF, 2016). Rivers and streams are negatively impacted by direct alteration (e.g. channelisation, damming), as well as land use/land cover (LULC) change of the terrestrial ecosystems within their watersheds (Abell et al., 2016; Collen et al., 2014). Deforestation and conversion of riparian zones to agricultural and urban land cover cause dramatic modifications in stream biogeochemistry, thermal regimes, hydrodynamics, and microhabitat structure due to changes in the quality and quantity of allochthonous inputs (Foster et al., 2003; Gardiner et al., 2009; Smucker et al., 2015). The resulting conditions may no longer be suitable for native species as physiological tolerance thresholds are crossed (Beitinger, Bennett, & McCauley, 2000; Davis, 1975; Nielsen, Brock, Rees, & Baldwin, 2003). However, impacts of altered conditions on species and populations may not be readily apparent if they are sub-lethal or result in delayed mortality (Budy, Thiede, Bouwes, Petrosky, & Schaller, 2002). Long-lived species are most likely to experience delayed extirpations following habitat loss or degradation (Krauss et al., 2010; Kuussaari et al., 2009; Morris et al., 2008), yet population declines of these species can be difficult to detect without long-term monitoring and the cause of decline is often difficult to ascertain post hoc (Gibbons et al., 2000; Maynou et al., 2011; Wheeler, Prosen, Mathis, & Wilkinson, 2003). The pace and extent of population declines coupled with the lack of long-term monitoring data necessitate that alternative population assessment methods be explored so that timely conservation actions can be implemented. Likewise, the multitude of potential agents of decline necessitates more holistic studies to evaluate the relative impacts of stressors that may be working at multiple spatio-temporal scales. Technological advances have led to the development and availability of rapid survey methods and improvements in freely available datasets that allow for more holistic, timely surveys. For example, the use of environmental DNA (eDNA) for detecting species is a relatively novel, cost-effective, non-invasive survey approach that allows for the rapid assessment of species distribution in a wide variety of ecosystems (Thomsen & Willerslev, 2015). The use of eDNA has not only been deemed reliable for establishing the presence of cryptic,

elusive, and rare species, but can surpass the sensitivity of traditional survey methods for detecting such species (Dejean et al., 2012; Goldberg, Pilliod, Arkle, & Waits, 2011; Olson, Briggler, & Williams, 2012; Pilliod, Goldberg, Arkle, & Waits, 2013; Spear, Groves, Williams, & Waits, 2015). Thus, its use for rapidly and effectively assessing and monitoring biodiversity has been recently highlighted as an emerging conservation tool (Thomsen & Willerslev, 2015). Similarly, technological advances have yielded high-quality (high resolution, fine scale) remote sensing datasets that allow for improved remote monitoring of a wide variety of ecological indicators (e.g. LULC change) and some species (Husson, Hagner, & Ecke, 2014; Pettorelli et al., 2014). The dissemination of information via the internet also presents new opportunities for biological monitoring. Online databases that compile species records have the potential to be used for monitoring biodiversity and species distribution (Miyazaki et al., 2014; Simpson et al., 2009). Integrating newer survey techniques and freely available data with traditional field work may allow for comprehensive, efficient, effective, and rapid assessment of changes in species distribution. The resulting data can be used to create more holistic models for assessing variables that may cause or serve as predictors of population declines and extirpations.

Our goal was to use detailed historical species records and eDNA survey methods to identify regional changes in population distribution of a long-lived (30+ years), imperiled stream salamander, the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*). The eastern hellbender exemplifies the need for amassing timely population data as they are currently being considered for listing under the U.S. Endangered Species Act with a decision regarding formal proposal submission to be made 30 September 2018 (J. Applegate, US Fish and Wildlife Service, personal communication). Hellbenders occupy cool, fast-flowing rivers and streams with high water quality and large rocks that they use for diurnal refugia and nesting habitat (Briggler, Utrup, et al., 2007; Nickerson, Krysko, & Owen, 2003; Nickerson & Mays, 1973; Pugh, Hutchins, Madritch, Siefferman, & Gangloff, 2016). They are excellent indicators of water quality and stream integrity due to their fully aquatic lifecycle, highly permeable skin, long life, and specialised habitat requirements (Nickerson & Mays, 1973; Nickerson et al., 2003). Population surveys revealed precipitous declines in many portions of their range, with some populations exhibiting signs of extinction debt such as ageing populations and lack of recruitment (Briggler, Ettling, Wanner, Schuette, & Ducan, 2007; Burgmeier, Unger, Sutton, & Williams, 2011; Foster, McMillan, & Roblee, 2009; Gates, Hocutt, Stauffer, & Taylor, 1985; Graham et al., 2011; Keitzer, Pauley, & Burcher, 2013; Pflingsten, 1990; Wheeler et al., 2003). Habitat degradation associated with LULC change continues to be a primary threat to hellbender populations (Briggler, Utrup, et al., 2007), but the relative contribution of various habitat changes to population decline is poorly understood. The distribution and status of hellbender populations in large portions of their range remain poorly known (Bodinof Jachowski, Millspaugh, & Hopkins, 2016), in part because traditional survey methods are time-consuming and labour-intensive and hellbenders can be difficult to detect (Nickerson & Mays, 1973; Rossell et al.,

2013). Several studies have successfully used eDNA to detect the presence of giant salamanders such as hellbenders (Fukumoto, Ushimaru, & Minamoto, 2015; Olson et al., 2012; Santas, Persaud, Wolfe, & Bauman, 2013; Spear et al., 2015). In particular, the quantitative PCR-based technique of Spear et al. (2015) for hellbenders has detected hellbenders at more sites than concurrent traditional survey techniques and allows for more rapid, large-scale, systematic surveys. We sought to use the population status data along with field-collected within-stream habitat data and reach- and watershed-scale LULC data derived from freely available, high resolution (1 m) remote sensing imagery to elucidate predictors of hellbender population extirpation and persistence in a region subjected to varying-intensity LULC change associated with agriculture, resource extraction, and urbanisation. We hoped our study would not only provide insight into the habitat variables associated with hellbender population persistence and extirpation, but also provide a framework for evaluating factors associated with changes in distribution of other understudied, cryptic, elusive, and rare aquatic species.

## 2 | METHODS

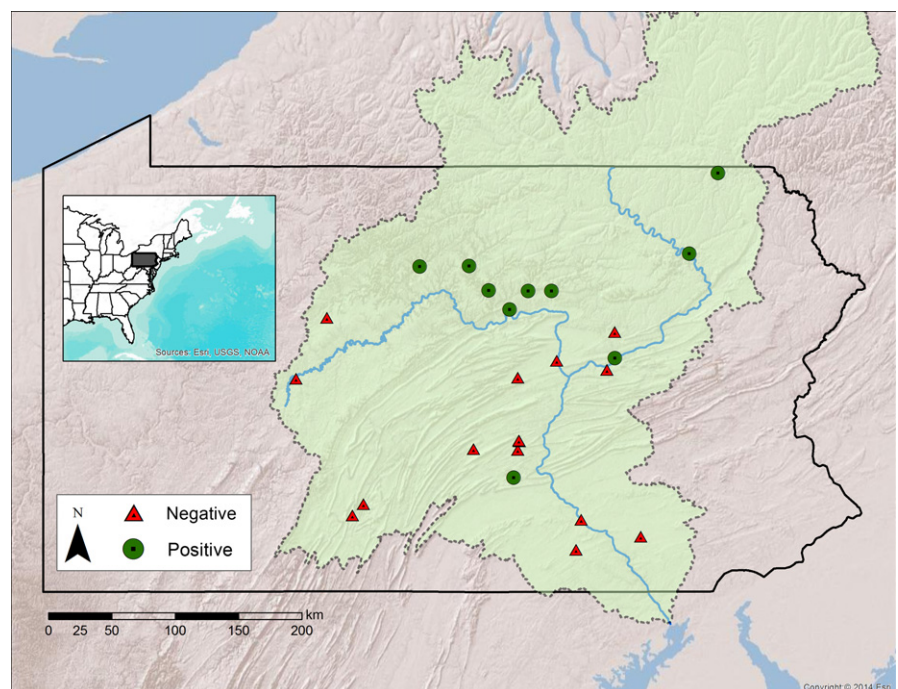
### 2.1 | Study area

Our study area encompassed streams within the Susquehanna River drainage of Pennsylvania (Figure 1). The Susquehanna River is an approximately 715 km long river that flows from Otsego Lake in New York to the Chesapeake Bay in Maryland (Pennsylvania Department of Conservation and Natural Resources, 2016). Its drainage encompasses 71,251 km<sup>2</sup>, 76% (54,286 km<sup>2</sup>) of which is located within Pennsylvania (Pennsylvania Department of Conservation and Natural Resources, 2016). Many historical eastern hellbender records

exist for this region and it was unclear whether this Pennsylvania Species of Greatest Conservation Need (SGCN; Pennsylvania Game Commission & Pennsylvania Fish & Boat Commission, 2015) has undergone range contractions within the state as it has elsewhere (Quinn, Gibbs, Hall, & Petokas, 2013).

### 2.2 | Historical data & study site selection

In order to specifically address the question of population persistence and extirpation, we used high-quality historical records to determine the location of documented hellbender populations. We collected eastern hellbender distributional accounts and records for the Susquehanna River drainage of Pennsylvania from museum records, primary literature, survey records, government reports, atlases, newspaper and magazine articles, and reputable personal communications (i.e. local ecological knowledge). Museum records were obtained by searching herpetological specimen databases provided on museum collection websites, as well as the HerpNet global herpetological collection database ([www.herpnet2.org](http://www.herpnet2.org); Now VertNet [www.vertnet.org](http://www.vertnet.org)). Primary literature, published anecdotal reports, and accounts in newspapers/magazines were obtained by searching primary literature databases (e.g. Thompson Reuters' Web of Science, PUBMED, JSTOR), internet search engines (e.g. Google Scholar, Google), and online newspaper databases. Search terms included "hellbender," "Pennsylvania," "Susquehanna," "*Cryptobranchus*," "fishing," "waterdog," "alligator" and the names of streams and counties within the Susquehanna River drainage of Pennsylvania. Additionally, we acquired local ecological knowledge regarding anecdotal accounts of hellbender occupancy within Susquehanna River drainage streams from reputable sources who could demonstrate that they could identify a hellbender from other species (e.g.



**FIGURE 1** Reassessed historic eastern hellbender (*Cryptobranchus alleganiensis*) sites within the Susquehanna River drainage of Pennsylvania, U.S.A. Dashed outline indicates Susquehanna River drainage boundary

mudpuppy, *Necturus maculosus*). We vetted historical accounts from newspaper and magazine articles based on the presence of pictorial or photographic evidence or detailed descriptions of the species to confirm the accounts were actually of *C. a. alleganiensis* and not alternative species that were reported under a misnomer or shared colloquial name. All remaining historical accounts were further vetted based on quality of location data. We only included sites with highly detailed location descriptions (e.g. latitude/longitude coordinates, landmark information) in our field surveys.

### 2.3 | Field surveys & sample processing

We conducted field surveys between 30 August and 29 September 2014, a time period that corresponds with the hellbender breeding season and low stream flow, thus increasing the probability of detecting hellbender eDNA (Spear et al., 2015). Because we wanted to determine if hellbender populations persisted at the reported historical locations, our goal was to collect samples from the same location as reported for the historical records. Often, historical records revealed precise locality data (e.g. latitude/longitude coordinates, location was marked on a map), hence most sampling was conducted at the exact location where hellbender occurrence was reported in the past. In the absence of precise locality data for a given stream, we used landmarks (e.g. bridges, stream confluences) noted in the records as reference for finding the sites. We sampled in areas that matched the location description provided, as well as in areas that appeared to be the best available hellbender habitat near the landmarks. Sampling locations were georeferenced using a Trimble Juno 5 handheld GPS (Trimble Navigation Limited, Sunnyvale, CA, U.S.A.).

We followed the eDNA survey protocol of Spear et al. (2015); a recent study estimated a detection probability of 0.9 for hellbender DNA using this specific protocol (Franklin, 2016). Reported detection probabilities for traditional snorkelling-based survey methods range up to 0.9 (Franklin, 2016; Pugh et al., 2016), but may be dramatically reduced by factors such as low visibility (Bodinof Jachowski et al., 2016). Furthermore, the use of eDNA has consistently identified more hellbender sites than traditional snorkelling-based survey methods alone (Franklin, 2016; Santas et al., 2013; Spear et al., 2015). For each site, we used new disposable cups to collect grab samples totaling 1 L of water. The sample collector entered the stream, maintaining a course perpendicular to the bank, until they reached the sampling location. Samples were collected upstream of the collector and their pathway in the stream, and before other members of the field team entered the stream to ensure samples were not contaminated. We used a Mityvac MV8010 Selectline Hand Pump (Lincoln Industrial Corp., St Louis, MO, U.S.A.) attached to a Nalgene Vacuum Flask (Thermo Fisher Scientific Inc., Rochester, NY, U.S.A.) to filter the water through Whatman Disposable Filter Funnels (particle retention maximum = 0.45  $\mu\text{m}$ ; GE Healthcare Bio-Sciences, Pittsburgh, PA, U.S.A.). Following filtration, filters were transferred to and stored in 95% ethanol in new 2.0 ml screw-cap vials. Equipment that was reused among sites (e.g. forceps used for filter transfer) was cleaned with DNA Away Surface Decontaminant (Molecular Bio-

products, Inc., San Diego, CA, U.S.A.) prior to each use to avoid sample cross-contamination. For quality assurance and quality control, we filtered deionised water after every fifth sample to serve as negative controls. For positive controls, we sampled sites with known persisting hellbender populations that had been recently field-verified by traditional survey techniques.

Laboratory methods followed that of Spear et al. (2015). Briefly, we extracted the DNA from the filters using a modified protocol of the DNeasy<sup>®</sup> Blood and Tissue Kit (Qiagen, Inc., Venlo, The Netherlands). We then amplified hellbender DNA with quantitative PCR using a species-specific primer and probe set and Qiagen QuantiTect Multiplex PCR Mix (Qiagen, Inc.). Each sample also included an internal positive DNA control (TaqMan Exogenous Internal Positive Control; ThermoFisher Scientific, Waltham, MA) that allowed us to detect whether any samples displayed full or partial PCR inhibition (McKee et al., 2015). Samples were run on an Applied Biosystems StepOne Plus Real-time PCR system (ThermoFisher Scientific, Waltham, MA). We included both extraction and PCR negative controls. Extraction and PCR setup was conducted in a facility dedicated to low copy DNA analysis at the Environmental Health Science Department at the University of Georgia. Samples were run in triplicate in the laboratory analyses. A sample was considered positive if two PCR replicates amplified. If only one of three replicates amplified in a single run, that sample was rerun and the sample was considered positive if at least one replicate amplified in the second run.

We measured within-stream habitat variables for each site. We used a Eureka multiprobe water quality meter (Eureka Water Probes, Austin, TX, U.S.A.) to measure water quality parameters including temperature, electrical conductivity, pH, dissolved oxygen (DO), and turbidity at the eDNA sampling site. Water quality parameters for all sites were collected within a five-day time period (13–17 September) to minimise impacts of seasonal and daily weather changes. We used a gravelometer (Wildlife Supply Co., Yulee, FL, U.S.A.) to estimate substrate composition in 10 regularly-spaced 30 cm by 30 cm quadrats along a transect that bisected each stream at the eDNA sampling site. Substrate was classified as silt/fine sediment, sand, gravel sizes 2–16, gravel sizes 22.6–90, gravel sizes  $\geq 128$ , rocks/boulders, bedrock and other (e.g. woody debris).

As pathogen transmission among populations is a major concern, we followed strict disinfection protocols of our persons and equipment between each sampling site. All equipment including waders was manually cleared of debris (e.g. mud, vegetation), disinfected (contact time  $\geq 5$  min) with a 10% bleach solution (used for waders and other hardy equipment) or 70% ethanol solution (used for sensitive equipment), then thoroughly rinsed with clean water between sampling sites. At the end of each sampling trip, all equipment was thoroughly washed in the lab in addition to the disinfection protocol.

### 2.4 | Spatial analysis

For each sample site, we evaluated canopy cover at different spatio-temporal scales. At the watershed scale, we evaluated total

canopy cover and riparian canopy cover. At the reach scale, we evaluated both recent (i.e. based on the most up-to-date, highest resolution imagery available) and historic (i.e. based on the nearest available imagery in time to documented historical presence) canopy cover. We chose to focus on canopy cover because forested watersheds and riparian zones effectively perform all ecological functions necessary for maintaining high-quality stream habitats (Barling & Moore, 1994; Gregory, Swanson, McKee, & Cummins, 1991; Jones et al., 2010; Vidon & Hill, 2004; Wenger & Fowler, 2000). Additionally, relative proportions of LULC classes within a given area are not independent and may confound analyses (King et al., 2005). To ensure accuracy of our canopy cover estimates, we used the highest resolution datasets available instead of the 30 m resolution data available from the National Land Cover Database (NLCD; <http://www.mrlc.gov/index.php>). While the NLCD is regularly used in ecological studies for estimating LULC at the reach and watershed scales, its use is known to result in inaccurate estimates due to the contrast of the study scale relative to the coarse resolution of the data (Nowak & Greenfield, 2010; Smucker et al., 2015).

We used ArcMap 10.3 (ESRI, Redlands, CA, U.S.A.) to manipulate and analyse spatial data. For watershed-scale analyses, we extracted the upstream watershed boundary for each sampling site using the U.S. Geological Survey (USGS) StreamStats online web app (<http://water.usgs.gov/osw/streamstats/>). We calculated total and riparian (150 m buffer) canopy cover for each watershed using a 1 m resolution canopy cover layer (University of Vermont Spatial Analysis Lab, 2015) clipped to each upstream watershed boundary and stream network (based on a stream layer from the National Hydrography Dataset; <http://nhd.usgs.gov>), respectively. We selected a riparian buffer width of 150 m because it represents an extent that may more effectively maintain water quality and habitat in streams with greater water flux (Sweeney & Newbold, 2014) and is inclusive of the minimum 30 m buffer width typically recommended for water quality protection (Wenger & Fowler, 2000).

Our reach scale extended 750 m upstream of the sampling point and consisted of 250 m riparian buffers along each side of the stream channel. A reach length of at least 10 channel widths is considered a useful scale over which to relate stream morphology to channel processes, response potential, and habitat characteristics (Montgomery & Buffington, 1997); 750 m represented a reach length of at least 10 channel widths for streams in our study. The 250 m riparian buffer represented an intermediate value for reach-scale assessment that is inclusive of the minimum required buffer for maintaining both water quality in at least small stream systems (Sweeney & Newbold, 2014) and habitat for semi-aquatic herpetofauna (Semlitsch & Bodie, 2003). We estimated reach-scale recent canopy cover for each site from 1 m resolution 2013 National Agriculture Imagery Program (NAIP) imagery (Natural Resources Conservation Science Geospatial Data Gateway <https://gdg.sc.egov.usda.gov/>). We estimated historical canopy cover at the reach scale for each sampling site to address potential legacy effects associated with historical LULC (Allan, 2004) using 1:20,000 scale (10 m

resolution), georeferenced, leaf-on aerial imagery (Penn Pilot <http://www.pennpilot.psu.edu/>).

## 2.5 | Statistical analyses

We calculated Pearson's rank correlation coefficients to identify highly correlated ( $r \geq 0.7$ ) variables. We removed a subset of highly positively correlated variables from further analyses. From the remaining suite of variables, we created biologically meaningful a priori models based on the natural history of hellbenders to determine which variables were the strongest predictors of population persistence (Table 1).

Preliminary analyses revealed quasi-complete separation in our data, thus we used logistic regression with Bayesian inference and a priori model selection to determine which variables were most important in predicting hellbender population persistence and extirpation. The Bayesian approach with a weakly informative prior stabilises regression coefficients without removing the strongest predictor from the models (Gelman, Jakulin, Pittau, & Su, 2009). This analysis requires that each input variable be standardised to a mean of 0 and a standard deviation of 0.5 to ensure a commonly-interpretable scale (i.e. the standardisation ensures the difference in scale among variables does not confound the analyses; Gelman et al., 2009), thus we transformed data accordingly. This analysis imposes  $t$  family prior distributions on the coefficients, and the Cauchy prior distribution, which we used, is specifically recommended (Gelman et al., 2009). Models were ranked based on calculated AIC values corrected for small sample size ( $AIC_C$ ).

We performed statistical analyses using R (R Development Core Team, 2012). We used the package "arm" to conduct the logistic regression analysis (Gelman et al., 2016).

## 3 | RESULTS

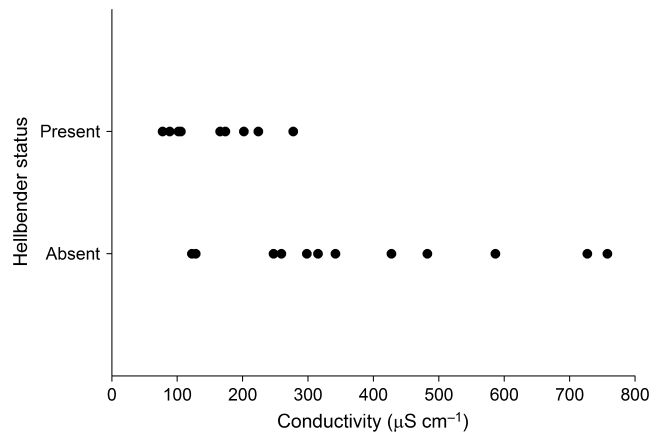
Historic records were available from as early as the late 1800s, and indicated hellbenders were widespread throughout the Susquehanna River drainage of Pennsylvania. Twenty-four historical records met our inclusion criteria (i.e. confirmed species identity and detailed location data), however, three sites were subsequently excluded from statistical analyses as the depth of the sites precluded assessment of the substrate. Ten of the 24 reassessed historical hellbender locations tested positive for hellbender eDNA, including all five of the sites that served as our positive controls (Figure 1). Five of the 10 positive samples amplified fully. All six field-processed negative control (i.e. deionised water) samples tested negative (0 of the replicates amplified) for hellbender eDNA. All three replicates of the laboratory extraction negative control also tested negative for hellbender eDNA. Of the 21 sites retained in the statistical analyses, nine tested positive for hellbender eDNA. The internal positive control amplified fully in each sample, indicating no evidence of PCR inhibition.

Total and riparian canopy cover at the watershed scale were very highly positively correlated ( $r = 0.96$ ,  $n = 21$ ,  $p < 0.001$ ), as were

**TABLE 1** Top five (of 10) best fit biologically meaningful a priori models assessed with logistic regression analysis with Bayesian inference and AIC model ranking corrected for small sample size (AICc) to determine which variables were the strongest predictors of eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) population persistence within the Susquehanna River drainage of Pennsylvania, U.S.A.

Model	AICc	Weight	Variables	$\beta$	SE	z	Pr(> z )	Odds ratio
Conductivity	22.7	0.417	Intercept	-0.7715	0.6427	-1.200	0.2300	
			Conductivity	-4.1675	1.8341	-2.272	0.0231*	0.015
RchDeforWQ	24.9	0.137	Intercept	-0.8779	0.6962	-1.261	0.2073	
			Recent reach canopy	-0.4297	1.0632	-0.404	0.6861	0.651
			Conductivity	-4.7076	2.1717	-2.168	0.0302*	0.009
WSDeforWQ	24.9	0.136	Intercept	-0.7401	0.6316	-1.172	0.2410	
			Watershed canopy	1.3036	1.3609	0.958	0.3380	3.683
			Conductivity	-3.0584	1.8624	-1.642	0.1010	0.047
WSCanopy	25.2	0.118	Intercept	-0.4635	0.5319	-0.871	0.3835	
			Watershed canopy	2.7774	1.2259	2.266	0.0235*	16.077
WithinStream	25.6	0.098	Intercept	-0.7250	0.6504	-1.115	0.2649	
			Conductivity	-3.8055	2.1538	-1.767	0.0773	0.022
			Sand/silt	-0.2708	1.1679	-0.232	0.8166	0.763

\*Statistical significance at  $\alpha = .05$ .



**FIGURE 2** Conductivity measures of stream sampling sites with persistent and extirpated eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) populations within the Susquehanna River drainage of Pennsylvania, U.S.A.

recent and historical reach canopy cover ( $r = 0.95$ ,  $n = 21$ ,  $p < 0.001$ ). Riparian canopy cover at the watershed scale was also highly positively correlated with gravel substrate ( $r = 0.71$ ,  $n = 21$ ,  $p < 0.001$ ). Conductivity was negatively correlated with total watershed canopy cover ( $r = -0.83$ ,  $n = 21$ ,  $p < 0.001$ ) and riparian canopy cover at the watershed scale ( $r = -0.77$ ,  $n = 21$ ,  $p < 0.001$ ).

The best fit model (AICc = 22.7) included only conductivity (Table 1; see also Appendix S1 in Supporting Information). Hellbender populations persisted in streams with conductivity values less than 278  $\mu\text{S}/\text{cm}$  (Figure 2).

## 4 | DISCUSSION

Historic records suggest that hellbenders occupied a wide distribution throughout the Susquehanna River drainage of Pennsylvania.

Our eDNA results suggest local extirpations and range constriction. Hellbenders persisted at only 42% of the 24 historical record sites. While there is always a possibility of false negatives with any survey method, the combination of high detectability (Franklin, 2016) and sampling during the season when eDNA concentrations should be elevated (Spear et al., 2015) give us confidence that our results represent the actual pattern of hellbender decline. Our results are consistent with those of others who revealed substantial declines and extirpations of hellbender populations in other regions (e.g. Briggler, Utrup, et al., 2007; Burgmeier et al., 2011; Foster et al., 2009; Gates et al., 1985; Graham et al., 2011; Keitzer et al., 2013; Pfungsten, 1990; Wheeler et al., 2003). These trends suggest eastern hellbenders warrant more intensive and comprehensive conservation action to ensure their persistence.

Our analyses indicate that conductivity is the strongest predictor of hellbender population persistence and extirpation. Hellbender populations persisted in sites with conductivity levels less than 278  $\mu\text{S}/\text{cm}$ , but conductivity of sampling sites ranged up to 758  $\mu\text{S}/\text{cm}$ . Conductivity is related to the total dissolved solids and specifically ion concentration in the water, and is known to be greater and more variable in streams in deforested landscapes (Likens, Bormann, Johnson, Fisher, & Pierce, 1970). Accordingly, in our study region, conductivity was strongly negatively correlated with total and riparian canopy cover at the watershed scale. However, because conductivity may also be affected by geophysical characteristics of streams and their settings (Liu, Weller, Correll, & Jordan, 2000), we completed a post hoc analysis in which we calculated Pearson's rank correlation coefficients to identify potential confounds. Conductivity was not strongly correlated with potentially confounding variables including stream order ( $r = 0.21$ ,  $n = 21$ ,  $p = 0.370$ ), elevation ( $r = -0.11$ ,  $n = 21$ ,  $p = 0.644$ ), and physiographic province ( $r = 0.32$ ,  $n = 21$ ,  $p = 0.155$ ). Thus, we conclude LULC change at the watershed scale is likely the initial driver of increased conductivity and

other water quality changes that more directly impact aquatic species. Other studies reported a negative correlation between conductivity and abundance of some salamanders (e.g. Bowles, Sanders, & Hansen, 2006; Willson & Dorcas, 2003) and occurrence of hellbenders (e.g. Bodinof Jachowski et al., 2016; Keitzer et al., 2013) in other regions, but conductivity has been dismissed as only an indicator of water quality decline. No direct connection between increased conductivity and hellbender population decline has been proposed in ecological studies. However, conditions leading to high conductivity (i.e. high ion concentration) may directly impact the reproductive success of aquatic species including salamanders and fish (Alavi & Cosson, 2006; Ettlting et al., 2013; Watanabe & Onitake, 2003). Sperm motility of aquatic-breeding salamanders and freshwater fish with external fertilisation is activated when sperm enter a hypo-osmotic (low solute and ion concentration) environment (Alavi & Cosson, 2006; Watanabe & Onitake, 2003). This mechanism of sperm activation appears to hold for hellbenders; Ettlting et al. (2013) initially observed low motility and deformities of sperm produced by male hellbenders that were maintained as breeding stock during the development of the St Louis Zoo's hellbender captive breeding program. However, after adjusting the ionic composition of the water, sperm were healthy and motile, resulting in successful fertilisation and reproduction (Ettlting et al., 2013). If high ion concentration (high conductivity) within streams inhibits hellbender sperm function, we would expect to see a lack of recruitment in declining hellbender populations. Because of the long lifespan of hellbenders, over time, this lack of recruitment would lead to populations typified by large, old individuals and few, if any, juveniles and subadults (i.e. smaller individuals). Declining populations in many portions of the hellbender range appear to be ageing populations typified by larger individuals and few individuals within smaller size (i.e. younger age) classes (Briggler, Utrup, et al., 2007; Burgmeier et al., 2011; Pugh et al., 2016; Wheeler et al., 2003), thus lending support to our hypothesis. It is ultimately expected that without conservation action, these populations would be extirpated as the older individuals die. Given that the eDNA survey methodology is not sufficient for assessing population size, nor does it provide information regarding population structure (Spear et al., 2015), we do not know if the persisting hellbender populations in our study area are viable or not. To address this shortcoming, we have initiated surveys using traditional survey techniques to evaluate the size and structure of a subset of the persisting populations. By evaluating population structure in conjunction with conductivity and other stream characteristics, we can add to our dataset by identifying stream characteristics associated with declining populations. These subsequent data will allow us to determine if the relationship revealed by our study holds for declining populations, as well as allow for the elucidation of populations in most critical need of conservation action.

Few other studies have considered conductivity in models evaluating hellbender occupancy and population status, and those that have, did not account for seasonal or weather-induced variability in conductivity and other water quality parameters in their sampling design. For example, Pugh et al. (2016) measured water quality

parameters in 21 sites once per year between June and August of 2011–13. Keitzer et al. (2013) measured water quality parameters in 58 sites once between May and November of 2006. Water quality parameters in a given area vary dramatically seasonally and with precipitation events, thus snapshots of water quality of various sites measured in different months or at different time periods relative to precipitation events may not be comparable (Kirchner, Feng, Neal, & Robson, 2004). Therefore, seasonal and weather-induced variation in water quality parameters may confound the results of analyses in which these data are used. While logistical constraints precluded our ability to measure water quality parameters in all sampling sites simultaneously, we minimised the influence of time and weather events by measuring all sites within a 5-day period during which it did not rain. Our study revealed that conductivity was the strongest predictor of hellbender population persistence and extirpation and we wonder if other studies may have yielded similar results if seasonal and weather-induced variability in water quality had been accounted for in their sampling design. Another benefit of our sampling design is that by measuring conductivity during the breeding season, our data may help to elucidate *in situ* conditions that promote or inhibit sperm function in our study region. However, we discourage the application of our conductivity measures to evaluate other regions because baseline conductivity measures (i.e. those not influenced by anthropogenic LULC) vary with underlying geology of different physiographic regions. For example, regions with karst (i.e. limestone and dolomite) geology should have relatively high baseline conductivity measures due to the ions that enter the water as the highly soluble rocks erode (Lamar & Shrode, 1953). Given the wide geographic distribution of hellbenders, it is likely that local adaptations have influenced the tolerance thresholds of individuals in different populations. For example, Keitzer et al. (2013) found that hellbenders were only present in locations with conductivity measures less than or equal to 53  $\mu\text{S}/\text{cm}$  in southern West Virginia. Conductivity measures of sites with persisting hellbender populations in our study area ranged from 77.7 to 277.4  $\mu\text{S}/\text{cm}$ . These differences may be related to local adaptations of hellbenders in each region, though caution should be implemented in comparing our data given the discrepancies in sample seasons between the two studies. We propose that evaluating the relative increase in conductivity above baseline levels within a given region is most meaningful for assessing suitability of habitats for supporting hellbenders as it better accounts for local adaptations.

By integrating historical data and eDNA survey techniques, our study design allows for relatively rapid assessment of changes in hellbender distribution over a relatively broad geographic area. Additionally, our analyses integrated more comparable water quality data, as well as LULC data obtained from high resolution imagery which is more appropriate for watershed and reach-scale analyses than the 30 m resolution NLCD imagery. Integrating eDNA survey techniques and freely available data with traditional field work allowed for comprehensive, efficient, effective, and rapid assessment of changes in hellbender distribution. This cost- and time-effective approach may be appropriate for evaluating distributional and habitat changes of

other understudied, cryptic, elusive, and rare aquatic species. The resulting data can be used to create more holistic models for assessing variables that can serve as predictors of population declines and extirpations.

## ACKNOWLEDGMENTS

Funding was provided by a Pennsylvania Wild Resource Conservation grant and a Bloomsburg University Research and Scholarship grant (both awarded to ALP while at Bloomsburg University). We are very grateful to J.D. Groves and L.A. Williams for providing eDNA sample collection training, L.F. Tucker Serniak, K. Cooper, R. Smollock, and M. Baade for field assistance, and S.A. Hartzell and R. Perch for sharing historic hellbender distribution information. We thank T. Glenn and E. Lipp for providing access to their lab space for eDNA analysis. We thank the two anonymous reviewers and Associate Editor B.O.L. Demars who provided helpful comments that led to the improvement of our manuscript. Research was approved by the Pennsylvania Fish and Boat Commission (Permit # 2014-01-083) and the Pennsylvania Department of Conservation and Natural Resources Bureau of State Parks (Permit # 2014-66).

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

**How to cite this article:** Pitt AL, Shinskie JL, Tavano JJ, Hartzell SM, Delahunty T, Spear SF. Decline of a giant salamander assessed with historical records, environmental DNA, and multi-scale habitat data. *Freshwater Biol.* 2017;00:1–10. <https://doi.org/10.1111/fwb.12917>